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Identification of Species Related to *Anopheles (Nyssorhynchus) albitarsis* by Random Amplified Polymorphic DNA-Polymerase Chain Reaction (Diptera: Culicidae)

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Species-specific Random Amplified Polymorphic DNA-Polymerase Chain Reaction (RAPD-PCR) markers were used to identify four species related to Anopheles *(*Nyssorhynchus*)* albitarsis *Lynch-Arribàlzaga from 12 sites in Brazil and 4 in Venezuela. In a previous study (Wilkerson et al. 1995), which included sites in Paraguay and Argentina, these four species were designated "A", "B", "C" and "D". It was hypothesized that species A is* An. *(*Nys.*)* albitarsis, *species B is undescribed, species C is* An. *(*Nys.*)* marajoara *Galvão and Damasceno and species D is* An. *(*Nys.*)* deaneorum *Rosa-Freitas. Species D, previously characterized by RAPD-PCR from a small sample from northern Argentina and southern Brazil, is reported here from the type locality of* An. *(*Nys.*)* deaneorum, *Guajará-Mirim, State of Rondônia, Brazil. Species C and D were found by RAPD-PCR to be sympatric at Costa Marques, State of Rondônia, Brazil. Species A and C have yet to be encountered at the same locality. The RAPD markers for species C were found to be conserved over 4,620 km; from Iguape, State of São Paulo, Brazil to Rio Socuavo, State of Zulia, Venezuela. RAPD-PCR was determined to be an effective means for the identification of unknown specimens within this species complex.*

Key words: *Anopheles (Nyssorhynchus) albitarsis -Anopheles (Nyssorhynchus) marajoara - Anopheles (Nyssorhynchus) deaneaorum* - RAPD PCR - identification

The Albitarsis Complex of *Anopheles* subgenus *Nyssorhynchus* (Albitarsis Subgroup of Linthicum 1988) is known to include a number of cryptic species (Kreutzer et al. 1976, Steiner et al. 1982, Rosa-Freitas et al. 1990, Narang et al. 1993). Most recently, using Random Amplified Polymorphic DNA - Polymerase Chain Reaction (RAPD-PCR), Wilkerson et al. (1995) demonstrated the existence of four species in the Albitarsis Complex in Brazil, Argentina and Paraguay. Members of this complex have been incriminated as important vectors of malaria parasites (reviewed by Rosa-Freitas et al. 1990) but, because members of the complex are difficult to identify by morphology, it is not known which species might be responsible for transmission in a given area. Our objective was to identify members of the Albitarsis Complex by RAPD-PCR from additional localities and obtain reared associated sibling voucher specimens for furture morphological studies. Becuase of its simplicity, the use of morphological characters is desirable as an aid to research in vector biology, biogeography, malaria epidemiology, malaria drug and vaccine trials and ultimately develop methodologies and strategies for malaria control. In addition, RAPD-PCR was investigated as a possible means for routine identifications in lieu of morphological identification keys.

At present, there are three valid species in the complex: *An. (Nys.) albitarsis* Lynch-Arribálzaga, 1878 (Paraguay, southern Brazil and Argentina); *An. (Nys.) deaneorum* Rosa-Freitas, 1989 (State of Rondônia, Brazil); and *An. (Nys.) marajoara* Galvão and Damasceno, 1942 (Costa Rica to Bolivia). Wilkerson et al. (1995) designated the taxa, characterized by RAPD-PCR, as species A, B, C and D. They hypothesized that species A is *An. (Nys.) albitarsis sensu stricto*, species B is undescribed, species C is *An. (Nys.) marajoara* and species D is *An. (Nys.) deaneorum.* RAPD genetic markers found by Wilkerson et al. (1995) were used to identify specimens collected at additional sites in Brazil and Venezuela. A summary of the distribution of the species and photo documentation of these markers is presented (Figs 1-16; Tables I-III).

The views of the authors do not purport to reflect the views of the supporting agencies.

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RAPD (Williams et al. 1990, Welsh & McClelland 1990) is a PCR-based technique which has proven to be a quick and effective means of identifying genetic markers. This technique permits numerous markers to be assayed on DNA extracted from a single mosquito. Instead of using primer pairs as in traditional PCR, RAPD reactions use a single short primer (usually 10 bases in length) of randomly chosen sequence. For a RAPD band to be produced, the primer needs to match a binding site that is within approximately 2-3 kilobase pairs of another oppositely oriented binding site so that the single oligonucleotide can prime replication in both the forward and reverse directions. A typical RAPD reaction produces multiple amplification products, each representing a discrete genetic locus, which can be resolved easily by agarose gel electrophoresis. RAPD-PCR has shown promise for use in a wide variety of organisms including bacteria, higher plants, vertebrates, and invertebrates including mosquitoes and other insects. It has been used as a tool for genetic mapping; strain, species and population identification and systematics (Hadrys et al. 1992, Chapco et al. 1992, Black et al. 1992, Kambhampati et al. 1992, Williams et al. 1993, Bowditch et al. 1993, Perring et al. 1993, Gawel & Bartlett 1993, Wilkerson et al. 1993, Wilkerson et al. 1995).

MATERIALS AND METHODS

Identification - Peyton et al. (1992) provide a definition of the subgenus *Nyssorhynchus*. Species in the Albitarsis Complex have hindtarsomere 5 without a basal dark band and vein 1A mostly pale-scaled (Argyritarsis Section) and sternum I with a distinct row of white scales on each side with posterolateral scale tufts beginning on segments III or IV. The sister species *An. braziliensis* (Chagas) has tufts beginning on segment II [see Linthicum (1988) for additional immature characters].

Specimen preservation - Adults and larvae used in DNA analysis were initially frozen in liquid nitrogen and transported to our laboratory on dry ice where they were maintained at -70°C. Larval and pupal exuviae were collected from individually reared adults and preserved in 80% ethanol and held for slide mounting. Each individually reared specimen was given a unique code number that associated it with other progeny of a single female and also associated it with a pin-mounted adult.

Source of specimens - Collection localities of F1 progeny broods used in this study are given in Table I along with those presented in Wilkerson et al. (1995). Associated specimens retained for morphogical study are deposited in the National Museum of Natural History, Smithsonian Institution, Washington DC; US Army Medical Research Unit, Rio de Janeiro; Instituto Oswaldo Cruz, Rio de Janeiro; and the Núcleo de Pesquisa Taxonômica e Sistemática em Entomologia Médica (NUPTEM) at the Universidade de São Paulo.

Experimental procedures - DNA Isolation. Individual larvae or adults were ground with a plastic pestle in microcentrifuge tubes in 200 µl extraction buffer (100mM Tris pH 8.0, 100mM EDTA, 100mM NaCl); proteinase K was then added to 200 µg/ml and SDS to 0.5%. After incubation at 55°C for 3 to 12 hr, RNase was added to a final concentration of 100 µg/ml and incubated at room temperature for 30 min. The solution was extracted once with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1, equilibrated with 10 mM Tris pH 8.0, 1mM EDTA) by heating to 55°C for 10 min with periodic mixing of phases. After brief centrifugation in a microcentrifuge, the supernatant was extracted with chloroform/isoamyl alcohol (24:1) as above. The supernatant was collected, 2 volumes (400µl) 95% ethanol were added to it, and the solution stored at - 20°C for 15 min to precipitate the DNA. The DNA was pelleted (15,900 x g in a microcentrifuge for 4 min), washed with 700 µl 70% ethanol, dried under vacuum, and dissolved in 100 µl 10 mM Tris pH 7.5, 1mM EDTA. Typical yields were 0.5 to 6.5 µg DNA per individual. *RAPD PCR amplification.* Detailed procedures are discussed in Bowditch et al. (1993). Total reaction volumes of 25 µl were used with the following final concentrations: 11 mM Tris-HCl (pH 8.3); 50 mM KCl; 1.9 mM MgCl2; 0.1 mg/ml BSA; 0.1 mM each of dATP, dCTP, dGTP and TTP; 0.24 pmol/µl (6 pmol) primer; 0.2-4.0 ng/µl template DNA; and 0.02-0.06 U/µl (0.5-1.5 units) *Taq* DNA polymerase. *PCR conditions.* A Perkin-Elmer Cetus model 480 thermocycler was used for all reactions with the following parameters: one min denaturation at 94°C followed by 45 cycles of one min at 94°C, annealing one min at 35°C and elongation two min at 72°C, all with minimum ramp times. *Agarose gel electrophoresis.* Using standard methods (Sambrook et al. 1989), amplification products were analyzed using a USA Scientific "Extra Wide" mini horizontal system (cat. no. 3488-0000) which uses 100 ml 1.5% agarose minigels with 0.8 µg/ml ethidium bromide run at 50 volts/25 milliamps for about 3 hr in TBE (89mM Tris base, 89mM boric acid and 2mM EDTA, pH 8.3). Amplification products were observed and photographed using short wave (312 nm) ultraviolet light. Molecular weight standards were provided by lambda DNA digested with *Hind* III, and ΦX174 DNA digested with *Hae* III (New England

Biolabs). The approximate molecular weight of amplification products was calculated using a program written for Lotus 1-2-3 by AF Cockburn, United States Department of Agriculture, Gainesville, Florida, USA. *Oligonucleotide primers.* All primers were 10 bases in length. Primers were purchased from Operon Technologies, Alameda CA. Primers discussed in the text have the following sequences (5'-3'): B16, TTTGCCCGGA; B05, TGCGCCCTTC; D01, ACCGCGAAGG; C07, GTCCCGACGA; B02, TGATCCCTGG; A01, CAGGCCCTTC; C19, GTTGCCAGCC; C16, CACACTCCAG; C15, GACGGATCAG; B11, GTAGACCCGT; A12, TCGGCGATAG; A08, GTGACGTAGG.

Based on the results of Wilkerson et al. (1995) (Figs 2-13) the following primers were used to amplify species-specific markers for identification of unknown specimens: Sp A, primers A01, B16, (2 markers); Sp B, primers C07, B02, C15 (4 markers); Sp C, primers A01, C16, C19, D01 (7 markers); and Sp D, primers A12, D01 (3 markers). In addition, the presence or absence of shared bands produced by primers B05 (species A,C), C19 (species A,B,D) and D01 (species C,D) were also taken into consideration (Table II). In order to make direct comparison with previous results, known specimens were run with all unknowns. To minimize the number of reactions needed for an identification, a "best guess" of a species identity was first made based on published and unpublished analyses of morphological characters [e.g. larval seta 3-C branched in *An. (Nys.) deaneorum* (Rosa-Freitas 1989) or length of basal dark band on hindtarsomere 2 and the relative length of the prehumeral dark spot (unpublished data)], or based on what is already known about the distributions of the species (e.g. species A is only recorded from southern Brazil, Bolivia, Paraguay and northern Argentina while species B has not been recorded from western Brazil). A species-specific primer was then used for the suspected species. Those matching were verified with one or two additional species-specific primers. Those that didn't match were tested with a next "best guess". Figure 14 shows species C primer A12 run with a series of specimens from Venezuela (lanes 4-18) and a single specimen from the State of Bahia, Brazil (lane 3). The known specimen of species C is in lane 2 from the type locality of *An. (Nys.) marajoara*, Ilha de Marajó, State of Pará, Brazil. The specimen in lane 3 that did not match was then run with species B primers with which it did match (not shown). Figure 15 shows a series of specimens from the type locality of *An. (Nys.) deaneorum*, Guajará-Mirim, State of Rondônia, Brazil (lanes 3-11 and 13) which, when run with species D primer A12, compared positively with a known species D from Guaíra, State of São Paulo, Brazil (lane 2). The specimens that did not match (lanes 12 and 14-19) from Peixoto de Azevedo, State of Mato Grosso, Brazil are among those run with species C primer C19 shown in Fig. 16 (lanes 3-9).

RESULTS

Based on genetic markers determined by Wilkerson et al. (1995) (Figs 2-13; Table II) we were able to identify the four known species belonging to the Albitarsis Complex from an additional 12 sites in Brazil and 4 in Venezuela (Table I; Fig 1). An additional eight individuals of species A, 41 of species B, 108 of species C and 26 of species D were identified. All (except nine species C) represent separate progeny broods with associated larval and pupal skins and pinned adults. To carry out identifications we used ten of the 12 primers used by Wilkerson et al. (1995) (Table II; Figs 2-13). These primers produced correlated markers as follow: A(3), D(3), B(4) and C(7). These data validate previous results (Wilkerson et al. 1995) with some variation (Table III).

Although it is preferable to use absolutely "fixed" markers for identifications, in reality this is probably not possible when one uses larger samples and/or widely separated populations. It

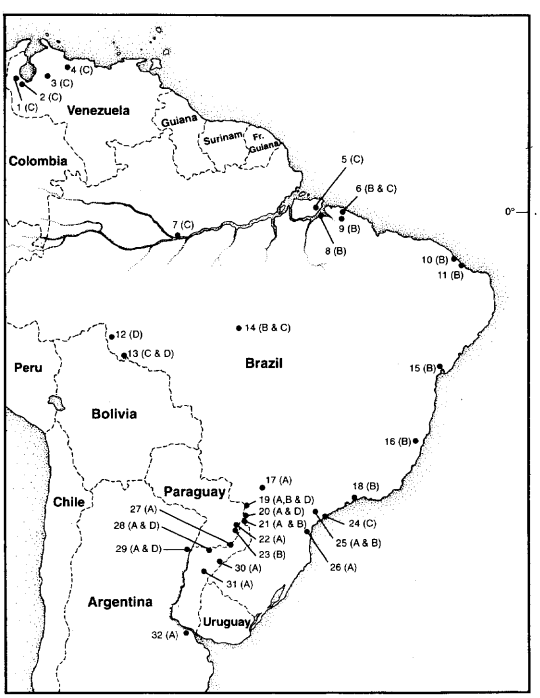

Fig. 1: summary of geographical distribution of the four known species in the Albitarsis Complex determined by RAPD-PCR. Presumed identities (see text) of species are as follow: A = *albitarsis*; B = undescribed; C = *marajoara* and; D = *deaneorum*.

TABLE I

Summary of collection localites for Albitarsis Complex species identified by Random Amplified Polymorphic DNA - Polymerase Chain Reaction

State/ Province	Locality	Coordinates	Date	Species(*n*)	Map Ref.
BRAZIL					
Rondônia[a]	Costa Marques	12°28'S 64°16'W	28-III-92	C(16), D(3)	13
Rondônia[a]	Guajará-Mirim	10°50'S 65°20'W	26-VII-92	D(23)	12
Pará[a]	Belém	1°27'S 48°29'W	8-X-92	B(2)	8
Pará[a]	Primavera	0°56'S 47°06'W	16-X-92	B(5)	9
Mato Grosso[a]	Matupá, Peixoto de Azevedo	10°23'S 54°54'W	20-IV-93	C(29), B(12)	14
Bahia[a]	Itaquara	13°26'S 39°56'W	30-I-93	B(9)	15
Ceará[a]	Fortaleza	3°43'S 38°30'W	8-III-93	B(11)	11
Ceará[a]	Parapaiba	3°25'S 39°13'W	9-III-93	B(13)	10
Santa Catarina[a]	Massaranduba	26°35'S 48°58'W	8-I-93	A(8)	26
Rio de Janeiro[a]	Morro da Panela	22°58'S 43°21'W	19-I93	B(1)	18
Amazonas[a]	Manaus	2°53'S 60°15'W	16-XII-93	C(34)	7
São Paulo	Ilha Comprida	24°42.8'S 47°31.6'W	9-II-89	C(18)	24
São Paulo	6 km SW Registro	24°36.8'S 47°53.1'W	26-I-92	A(30), B(19)	25
São Paulo	Ponte Melo Peixoto	22°39.05'S 53°01'W	10-II-92	A(1)	17
Pará	Capanema	1°24'S 47°11'W	VIII-93	C(8), B(1)	6
Pará	Ilha de Marajó	1°00'S 49°30'W	VIII-91	C(43)	5
Paraná	Santa Helena	24°56'S 54°23'W	30-I-92	A(5), D(1)	20
Paraná	nr. Guaíra	24°04'S 54°15'W	1-II-92	A(12), B(1), D(4)	19
Espírito Santo	Águia Branca	18°59'S 40°44'W	20-I-92	B(1)	16
VENEZUELA					
Zulia[a]	Rio Socuavo	8°54'N 72°38'W	22-IX-92	C(16)	1
Zulia[a]	Tres Bocas	?8°54'N 72°38'W	24-IX-92	C(1)	2
Cojedes[a]	Finca Rosa Blanca	9.6°N 68.9°W	26-VI-91	C(10)	4
Barinas[a]	Castilla	8°25'N 70°38'W	14-IX-92	C(2)	3
PARAGUAY					
Alto Paraná	Rio Acaray	25°29'S 54°42'W	4-II-92	A(9)	22
Alto Paraná	Hernandarias	25°22'S 54°45'W	6-II-92	A(8), B(6)	21
Alto Paraná	nr. National Airport	(not known)	8-II-92	B(9)	23
ARGENTINA					
Misiones	Posadas	27°23'S 55°53'W	30-I-92	A(12)	27
Corrientes	90km W Posadas	(not known)	31-I-92	A(1), D(2)	28
Corrientes	Corrientes	27°28'S 59°50'W	31-I-92	A(9), D(2)	29
Corrientes	11km W Mercedes	29°12'S 58°05'W	1-II-92	A(5)	31
Corrientes	Santa Tome	28°33'S 56°03'W	2-II-92	A(8)	30
Buenos Aires	Baradero	33°48'S 59°30'W	6-II-92	A(22)	32

a: collection localities presented here for the first time, others appear in Wilkerson (1995).

is not critical for the effective use of RAPD markers, to have100% fixed markers, but to have nearly fixed markers that are correlated. As long as all the exceptional missing markers do not correspond in a single individual, there should be no confusion about assigning individuals to their respective genetically defined taxa. A comparison of the frequency of diagnostic markers between Wilkerson et al. (1995) and the present study show some differences. The 0.38 kbp band produced by

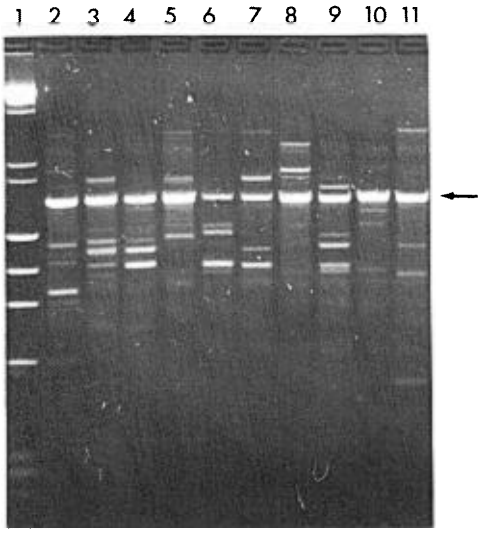

Fig. 2: RAPD amplifications of Albitarsis Complex species A with primer B16. Arrow on right indicates fragment of approximately 1.75 kbp. Lane 1, DNA size standard; 2-3, Baradero, Argentina; 4, Registro, Brazil; 5, Santa Tome, Arg.; 6, Posadas, Arg.; 7, Corrientes, Arg.; 8, Mercedes, Arg.; 9, near Santa Helena, Brazil; 10, near Guaira, Brazil; 11, Ponte Melo Peixoto, Brazil.

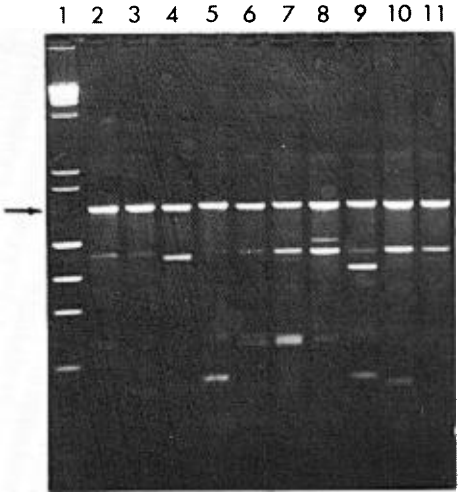

Fig. 3: RAPD amplifications of Albitarsis Complex species A with primer B05. Arrow on left indicates fragment of approximately 1.75 kbp. Lane 1, DNA size standard; 2-3, Baradero, Argentina; 4, Registro, Brazil; 5, Santa Tomé, Arg.; 6, Posadas, Arg.; 7, Corrientes, Arg.; 8, Mercedes, Arg.; 9, near Santa Helena, Brazil; 10, near Guaira, Brazil; 11, Ponte Melo Peixoto, Brazil.

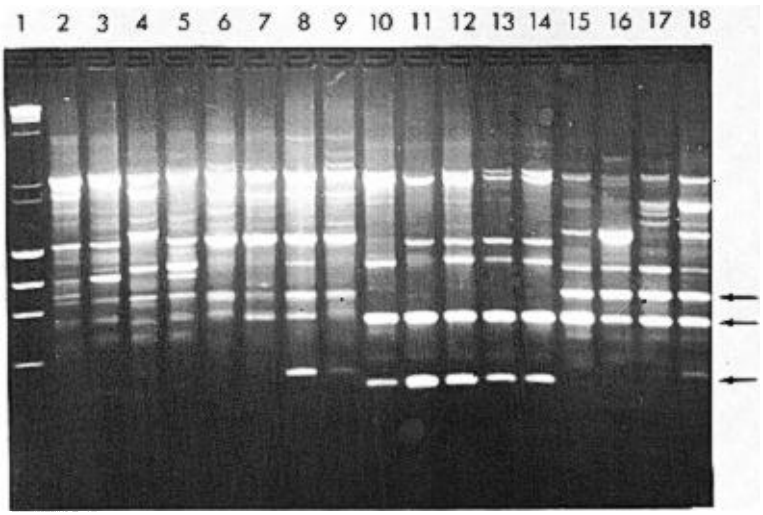

Fig. 4: RAPD amplifications of Albitarsis Complex species with primer D01. Lanes 2-5, species A; 6-9, species B; 10-14, species C; 15-18, species D. Arrows on left indicate fragments of approximately 2.32 kbp (all species) and 1.42 kbp (species A and B); arrows on right indicate fragments of approximately 0.96 kbp (species D), 0.80 kbp (species C and D) and 0.55 kbp (species C). Lane 1, DNA size standard (see Fig. 2); 2-3, Baradero, Argentina; 4, 6-7, Registro, Brazil; 5, 17, 18, near Guaira, Brazil; 8-9, 15, Hernandarias, Paraguay; 10-12, Ilha de Marajó, Brazil; 13-14, Iguape, Brazil; 16, 90 km W Posadas, Arg.

primer C07 and the 1.88 kbp band produced by primer B02 were missing in a small percentage of specimens reported here for the first time. Also, the 1.41, 1.22 and 0.85 kbp bands produced by primer A01 for species C were found in slightly lower frequencies than before. Wilkerson et al. (1995) reported that only one individual out of 61 had two of the three bands missing. In the present study, two of 108 also lacked these same two bands (1.22 and 0.85 kbp). However, the other four species C markers tested for by primers C16, D01 and C19 were present in both individuals.

The 1.00 kbp marker produced by primer C15 in species B was the only one found to be absent in a significant proportion of individuals compared to the previous study (100%, 15/15 vs 68.3%, 28/41). In addition, a 1.41 kbp band produced by this primer which was not recognized as a poten-

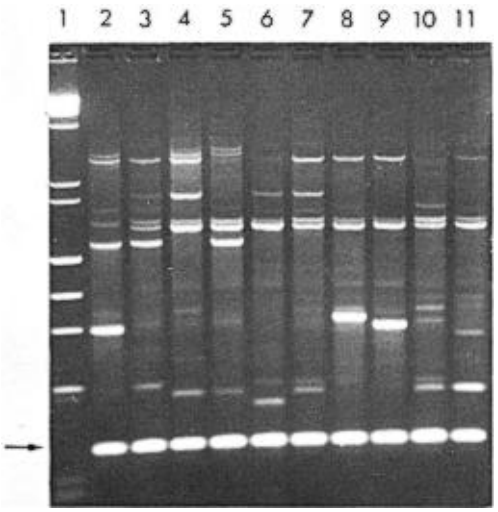

Fig. 5: RAPD amplifications of Albitarsis Complex species B with primer C07. Arrow indicates fragment of approximately 0.41 kbp. Lane 1, DNA size standard (see Fig. 2); 2-5, Registro, Brazil; 6-10, Hernandarias, Paraguay; 11, Águia Branca, Espírito Santo, Brazil.

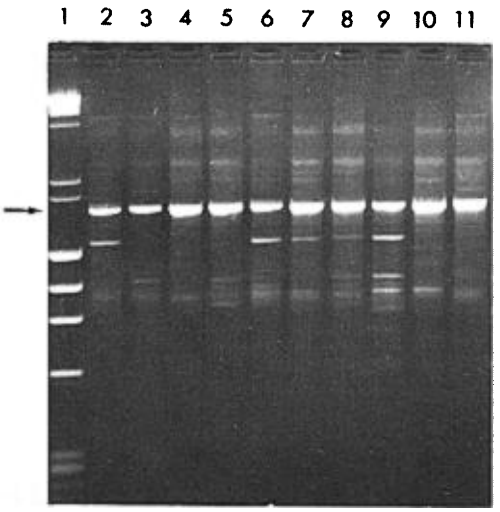

Fig. 6: RAPD amplifications of Albitarsis Complex species B with primer B02. Arrow indicates fragment of approximately 1.88 kbp. Lane 1, DNA size standard (see Fig. 2); 2-5, Registro, Brazil; 6-10, Hernandarias, Paraguay; 11, Águia Branca, Espírito Santo, Brazil.

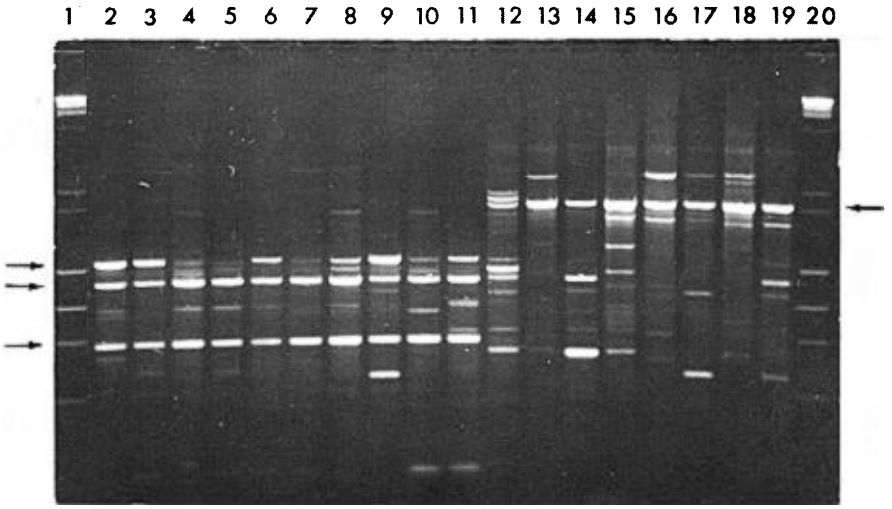

Fig. 7: RAPD amplifications of Albitarsis Complex species with primer A01. Lanes 2-11, species C; 12-19, species A. Arrows on left indicate fragments of approximately 1.41, 1.22 and 0.85 kbp (species C); arrow on right indicates fragment of approximately 2.03 kbp (species A). Lanes 1, 20, DNA size standard (see Fig. 2); 2-11, Iguape, Brazil; 12-15, Baradero, Argentina; 16-17, Registro, Brazil; 18-19, Hernandarias, Paraguay.

tially useful species-specific marker because of its inconsistent brightness, was actually consistently present and recognizable in all individuals tested. A possible homologous 1.41 kbp band was seen in four of fifteen species C, but further testing was not done to verify its size.

Twenty-three progeny broods from the type locality of *An. (Nys.) deaneorum* matched for all three markers previously determined for species D. Even though the initial species D sample from northern Argentina and southern Brazil was small (n = 9), these markers were also present in the larger sample (n = 26) from two localities in the State of Rondônia, Brazil.

A distance of approximately 2,400 km was previously reported (Wilkerson et al. 1995) between populations of species C. With the addition of specimens of species C from Venezuela, the maximum distance we have found between populations exhibiting the same diagnostic markers is now approximately 4,620 km (Iguape, State of São Paulo, Brazil to Rio Socuavo, State of Zulia, Venezuela).

Species C and D were sympatric at Costa

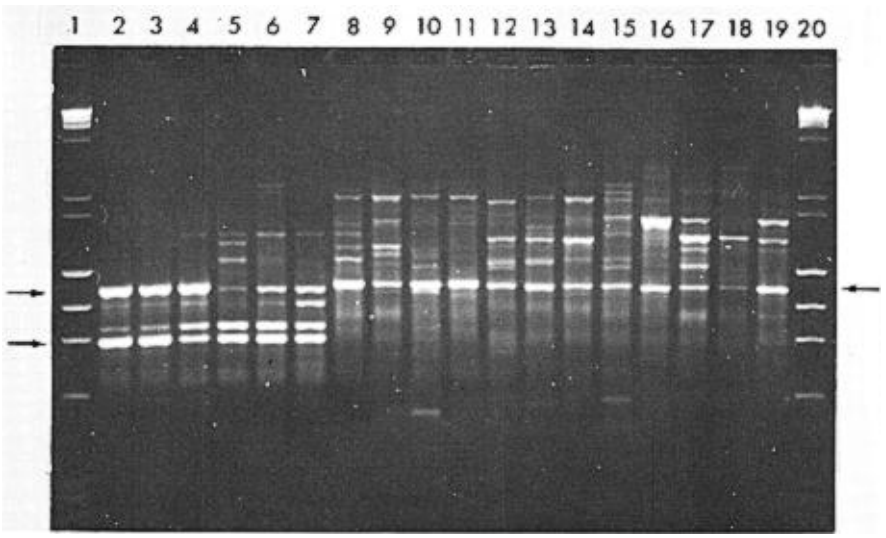

Fig. 8: RAPD amplifications of Albitarsis Complex species with primer C19. Lanes 2-7, species C; 8-11, species A; 12-15, species B; 16-19, species D. Arrows on left indicate fragments of approximately 1.172 and 0.88 kbp (species C); arrow on right indicates fragment of approximately 1.30 kbp (species A, B and D). Lanes 1 and 20, DNA size standard (see Fig. 2); 2-4, Ilha de Marajó, Brazil; 5-7, Iguape, Brazil; 8, Posadas, Argentina; 9, Baradero, Arg.; 10-13, Registro, Brazil; 14-15, Hernandarias, Paraguay; 16-17, 90 km W Posadas, Arg.; 18-19, near Guaíra, Brazil.

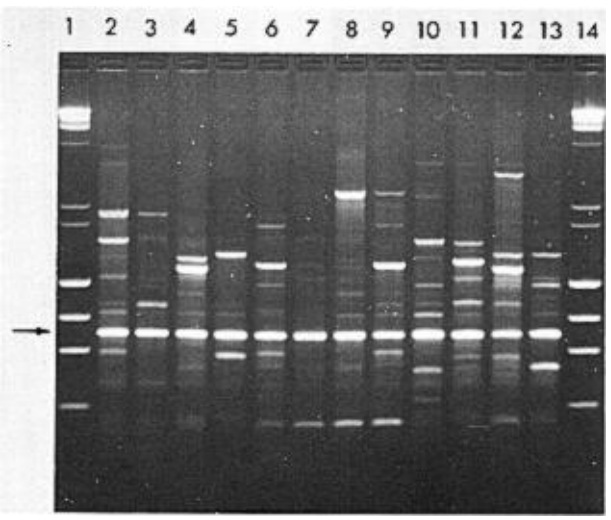

Fig. 9: RAPD amplifications of Albitarsis Complex species C with primer C16. Arrow indicates fragment of approximately 0.993 kbp. Lanes 1 and 14, DNA size standard (see Fig. 2); 2-5, Ilha de Marajó, Brazil; 6-9, Iguape, Brazil; 10-13, Capanema, Brazil.

Marques, State of Rondônia, Brazil. These two species had been collected previously in the same human and bovine bait collections (Klein & Lima 1990). With this finding, all possible pairs of species, except species A and C, have been found to be sympatric at some locality. The site near Guaíra, State of Paraná, Brazil remains the only site sampled with three of the species occurring together (A, B, D).

DISCUSSION

Using RAPD-PCR we have begun to solve parts of the Albitarsis Complex puzzle. However, the relation of most previous epidemiological, biological and morphological studies to our results cannot be determined with certainty (also discussed in Wilkerson et al. 1995). Many have attempted to correlate malaria transmission with behavior and/or relative length of the dark portion of hindtarsomere 2 (reviewed by Rosa-Freitas & Deane 1989 and Rosa-Freitas et al. 1990). Some of the studies demonstrated a correlation, while most were ambiguous. These attempts had their basis in early observations regarding malaria trans-

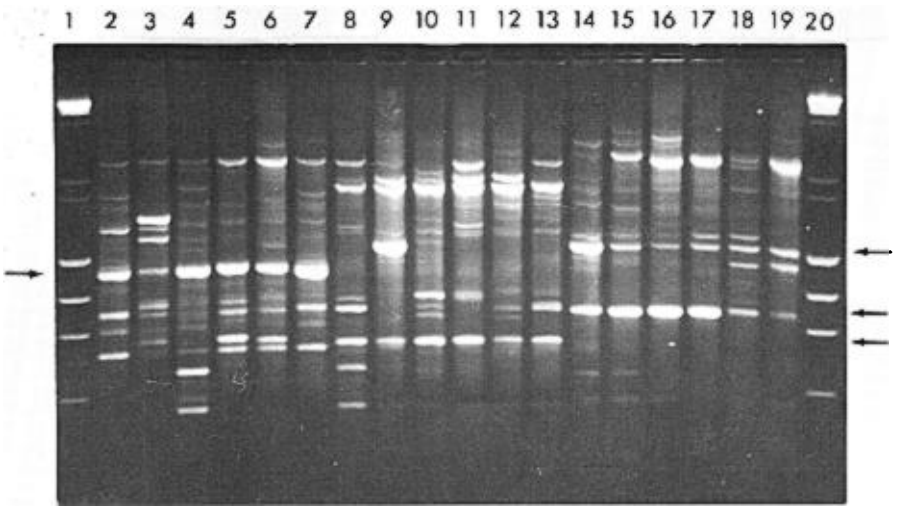

Fig. 10: RAPD amplifications of Albitarsis Complex species with primer C15. Lanes 2-7, species D; 8-13, species A; 14-19, species B. Arrow on left indicates fragment of approximately 1.24 kbp (species D); arrows on right indicate fragments of approximately 1.00 kbp and 1.41 kbp (species B) and 0.86 kbp (species A). Lanes 1 and 20, DNA size standard (see Fig. 2); 2-3, 90 km W Posadas, Argentina; 4, 6, 9, Corrientes, Arg.; 5, 13, near Guaíra, Brazil; 7, 12, near Santa Helena, Brazil; 8, Posadas, Arg.; 10, Baradero, Arg.; 11, 14-16, Registro, Brazil; 17-19, Hernandarias, Paraguay.

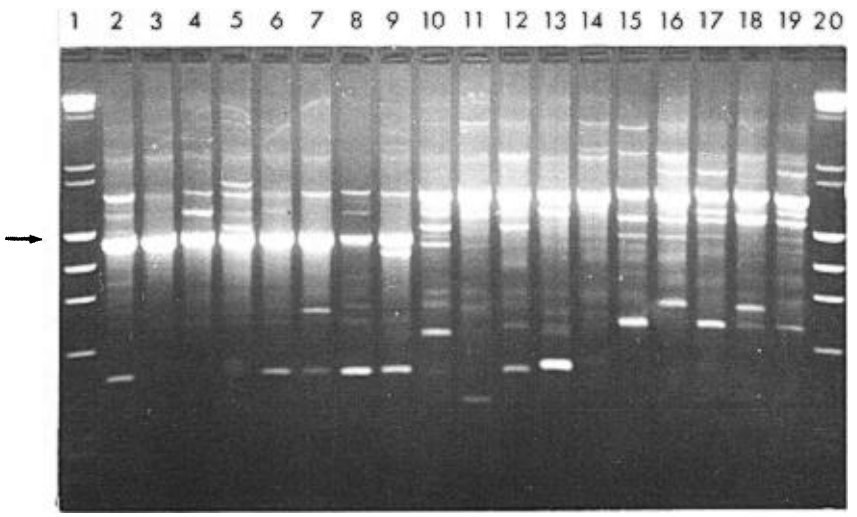

Fig. 11: RAPD amplifications of Albitarsis Complex species with primer B11. Lanes 2-9, species D; 10-14, species A; 15-19, species B. Arrow on left indicates fragment of approximately 1.26 kbp (species D). Lanes 1 and 20, DNA size standard (see Fig. 2); 2-3, 90 km W Posadas, Argentina; 4, 7-9, 14, near Guaíra, Brazil; 5, Corrientes, Arg.; 6, near Santa Helena, Brazil; 10, Posadas, Arg.; 11, Mercedes, Arg.; 12, Baradero, Arg.; 13, 15-16, Registro, Brazil; 17, Águia Branca, Espírito Santo, Brazil; 18-19, Hernandarias, Paraguay.

mission and biting-resting behavior. Galvão and Damasceno (1944) regarded *An. (Nys.) albitarsis albitarsis* as exophilic, not related to malaria transmission and to have 77-84% of hindtarsomere 2 dark. The only specimens we have seen that agree with this description of hindtarsomere 2 are described here as species A, "true" *An. (Nys.) albitarsis*. At the same time, Galvão and Damasceno (1944) described as new *An. (Nys.) albitarsis domesticus*, regarding it as endophilic, related to malaria transmission and to have 36-50% of hindtarsomere 2 dark. As a basis for their description they contrasted *domesticus* to *An. (Nys.) albitarsis albitarsis*. *An. (Nys.) albitarsis domesticus* was later synonymized under *An. (Nys.) marajoara* by Linthicum (1988). Since the type locality for both species is the same, and because no type specimens apparently exist for *An. (Nys.) albitarsis domesticus* to refute this, we agree with Linthicum's synonymy (previously reported in

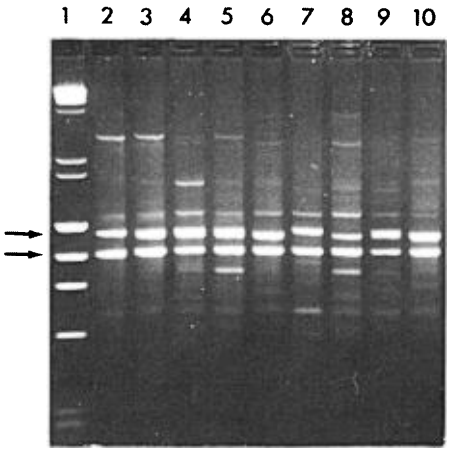

Fig. 12: RAPD amplifications of Albitarsis Complex species D with primer A12. Arrow on left indicate fragment of approximately 1.09 kbp. Lane 1, DNA size standard (see Fig. 2); 2-3, 90 km W Posadas, Argentina; 4, 6, Corrientes, Arg.; 5, 8-10, near Guaíra, Brazil; 7, near Santa Helena, Brazil.

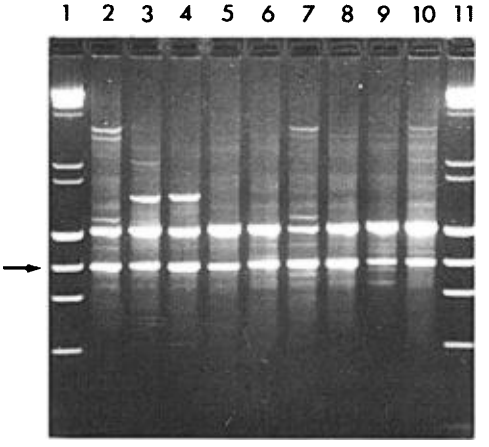

Fig. 13: RAPD amplifications of Albitarsis Complex species D with primer A08. Arrows on left indicate fragments of approximately 1.41 and 1.09 kbp. Lane 1, DNA size standard (see Fig. 2); 2-3, 90 km W Posadas, Argentina; 4, 6, Corrientes, Arg.; 5, 8-10, near Guaíra, Brazil; 7, near Santa Helena, Brazil.

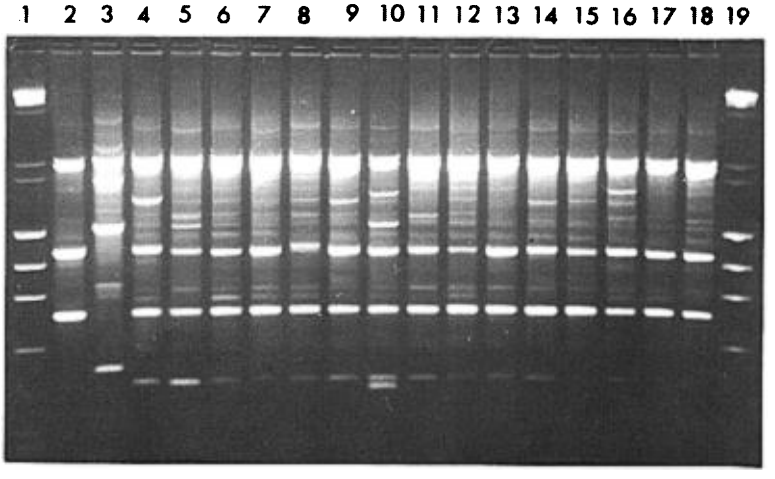

Fig. 14: screen of unknown Albitarsis Complex specimens with species C primer A12. Lanes 1 and 19, DNA size standard (see Fig. 2); lane 2, Ilha de Marajó, Pará, Brazil, type locality of *An. (Nys.) marajoara*; lane 3, Itaquara, Bahia, Brazil; lanes 4-18, Rio Socuavo, Zulia, Venezuela. When the specimen from Itaquara was run with species B primers C07, B02 and C15, it matched other specimens of species B.

Wilkerson et al. 1995). Even though there is a temptation to use such a useful descriptive name as "domesticus" for members of this complex that enter houses and transmit malaria, it should no longer be used since there may be several species which fit this description, to which none the name "domesticus" can be applied. After sampling 18 widely separated populations in Brazil, Rios et al. (1984) also came to the conclusion that it was impossible to separate the two putative subspecies, *An. (Nys.) albitarsis albitarsis* and *An. (Nys.) a. domesticus*, using these same "traditional" characters.

Anopheles (Nys.) marajoara sensu Linthicum (1988) is based primarily on specimens from Panama, not the type locality, Ilha de Marajó, Brazil. He included all specimens that he studied, except *An. (Nys.) albitarsis s.s.*, ranging from Costa Rica to southern Brazil, under the name *An. (Nys.) marajoara*, including those that were later described as *An. (Nys.) deanorum*. His discussion points out the great variation he found which strongly suggests that he was dealing with a mixture of taxa.

Previous cytogenetic and molecular studies

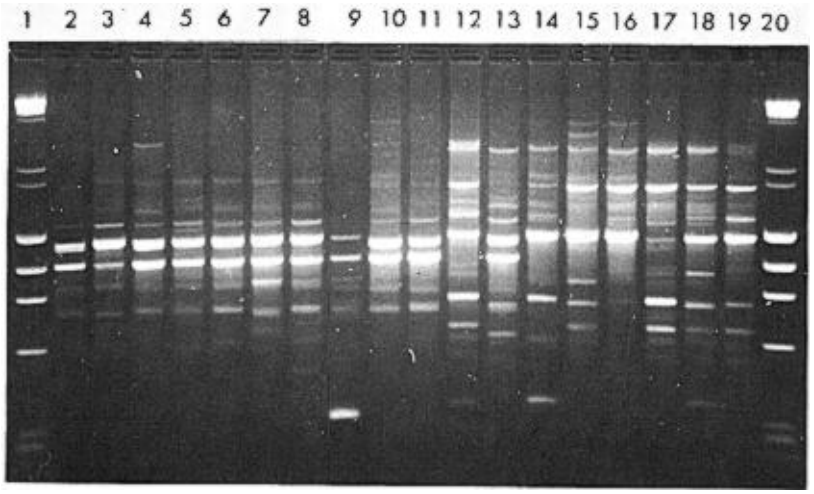

Fig. 15: screen of unknown Albitarsis Complex specimens with species D primer A12. Lanes 1 and 20, DNA size standard (see Fig. 2); Lanes 3-11, and 13, Guajará-Mirim, Rondônia, Brazil, type locality of *An. (Nys.) deaneorum;* lanes 12 and 14-19, Peixoto de Azevedo, Mato Grosso, Brazil.

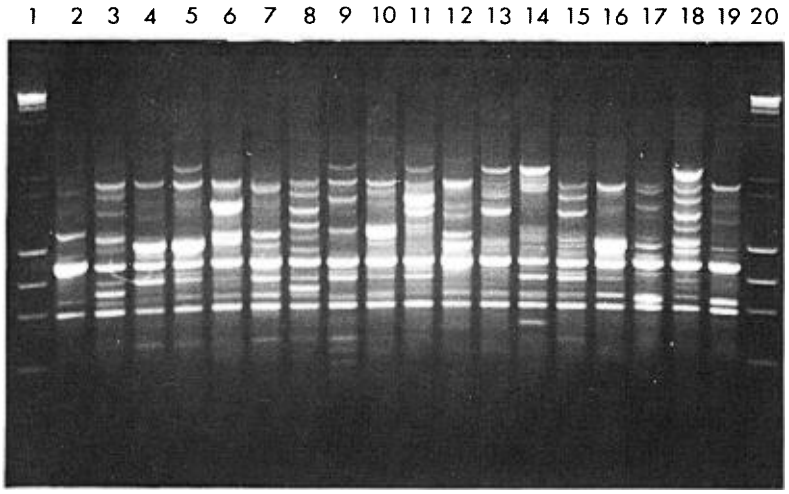

Fig. 16: screen of unknown Albitarsis Complex specimens with species C primer C19. Lanes 1 and 20, DNA size standard (see Fig. 2); lane 2, Ilha de Marajó, Para, Brazil, type locality of *An. (Nys.) marajoara*; lanes 3-19, Peixoto de Azevedo, Mato Grosso, Brazil. Includes specimens not matching markers for species D primer A12 given in Fig. 15.

have offered some insight into understanding this complex. It is not clear how these findings relate to those presented here because in most cases voucher specimens are not available for comparison. Kreutzer et al. (1976) found three chromosomally differentiated populations, "C" in Colombia and Venezuela and two others, B_1 and B_2, ranging from Rio de Janeiro to Belém and just north of Ilha de Marajó. We do not believe that we have yet sampled their population "C". Steiner et al. (1982), using isozyme analysis, found three "genetically differentiated" populations at sites from São Paulo to just west of Marajó Island based on 12 allozyme loci and chromosome analysis. According to their map, species "a" and "b" were found near the coast and "b" was also found inland but, according to their Table II, "a" was also found with "b" inland at Logoa (sic, Lagoa?) Feia. An additional discrepancy failed to show species "b" at Araraquara, sympatric with their species "c". These possibly correspond to "B_1" and "B_2" of Steiner et al. (1982). However, we were not able to locate published information on chromosomes cited in their analysis. In a study of 10 populations Rosa-Freitas et al. (1990) found no morphological differences, except for *An. (Nys.) deaneorum*, but through isoenzyme analysis concluded that there were five "groups" in Brazil and Argentina. Narang et al. (1993) compared four populations in the Albitarsis Complex in Brazil

TABLE II

Summary of Randon Amplified Polymorphic DNA markers used to identify species of the Albitarsis Complex

Primer	Approximate kbp	A	B	C	D
B16	1.75	+[a]	–	–	–
B05	1.74	+	–	+	–
D01	2.2-2.3	+	+	+	+
	0.96	–	–	–	+[a]
	0.80	–	–	+	+
	0.55	–	–	+[a]	–
C07	0.38	–	+[a]	–	–
B02	1.88	–	+[a]	–	–
A01	2.03	+[a]	–	–	–
	1.41	–	–	+[a]	–
	1.22	–	–	+[a]	–
	0.85	–	–	+[a]	–
C19	1.30	+	+	–	+
	1.17	–	–	+[a]	–
	0.88	–	–	+[a]	–
C16	0.99	–	–	+[a]	–
C15	1.41	–	+[a]	–	–
	1.24	–	–	–	+[a]
	1.00	–	+[a]	–	–
	0.86	+[a]	–	–	–
B11	1.78	+	+	+	–
	1.26	–	–	–	+[a]
A12	1.29	–	–	–	+[a]
	1.09	–	–	–	+[a]
A08	1.09	–	–	–	+[a]

a: species specific markers; +: marker in common to more than one species; –: marker not present species

using 18 allozyme loci and mitochondrial DNA restriction fragment length polymorphisms. They reported that two morphologically distinct taxa existed, *An. (Nys.) deaneorum* and *An. (Nys.) marajoara*, the latter made up of 2 or 3 biochemically recognizable forms. One of the forms of *An. (Nys.) marajoara* was sympatric with *An. (Nys.) deaneorum* at Costa Marques, State of Rondônia, and the other one or two forms of *An. (Nys.) marajoara*, depending on interpretation, were from Iguape, State of São Paulo, and from its type locality of Marajó Island, State of Pará. The Iguape specimens are the same as those used by us in the present study (species C) and may represent a species responsible for a malaria outbreak in Iguape reported by Schiavi (1945). As reported here the two species from Costa Marques (C, D) are probably *An. (Nys.) marajoara* and *An. (Nys.) deaneaorum*.

RAPD-PCR has proven to be effective in separating cryptic *Anopheles* species and for identifying unknown specimens. It has the potential for use as a routine diagnostic tool for identification of *Anopheles* sibling species, but because of the sensitivity of RAPD-PCR to changing experimental conditions, care should be taken in its use (Williams et al. 1993, Black 1993). It has the advantage of relatively low initial investment of time for determining species-specific markers when compared to designing and testing custom-made species-specific PCR primers based on known nucleotide sequences.

TABLE III

Diagnostic primers used for identification of Albitarsis Compelex species and comparison to Wilkerson (1995) results

Species	Primer	Appox. kbp	Wilkerson (1995)	Present study
A	B05	1.74	121/121 (100%)	8/8 (100%)
	B16	1.75	81/82 (98.8%)	8/8 (100%)
	A01	2.03	118/118 (100%)	8/8 (100%)
B	C07	0.38	32/32 (100%)	40/41 (97.5%)
	B02	1.88	35/35 (100%)	38/41 (92.6%)
	C15	1.00	15/15 (100%)	28/41 (68.3%)
		1.41	15/15 (100%)	41/41 (100%)
C	C16	0.99	63/63 (100%)	66/66 (100%)
	D01	0.55	42/42 (100%)	29/29 (100%)
	A01	1.41	59/61 (96.7%)	91/108 (84.3%)
		1.22	58/61 (95.1%)	86/108 (79.6%)
		0.85	59/61 (96.7%)	100/108 (92.3%)
	C19	1.17	58/58 (100%)	84/84 (100%)
		0.88	58/58 (100%)	84/84 (100%)
D	A12	1.29	9/9 (100%)	26/26 (100%)
		1.09	9/9 (100%)	26/26 (100%)
	D01	0.96	9/9 (100%)	26/26 (100%)

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